Received: 12 September 2018 | Revised: 21 May 2019 | Accepted: 24 May 2019

DOI: 10.1002/term.2912

RESEARCH ARTICLE

WILEY

Contribution of the WNK1 kinase to corneal wound healing using the tissue-engineered human cornea as an in vitro model

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Funding information
Réseau de thérapie cellulaire, tissulaire et génique (ThéCell); Fonds de Recherche du Québec-Santé (FRQS); Fondation des Pompiers du Québec pour les Grands Brûlés; Canadian Institutes for Health Research (CIHR), Grant/Award Number: MOP-53170

Abstract

Damage to the corneal epithelium triggers important changes in the extracellular matrix (ECM) to which basal human corneal epithelial cells (hCECs) attach. These changes are perceived by integrin receptors that activate different intracellular signalling pathways, ultimately leading to re-epithelialization of the injured epithelium. In this study, we investigated the impact of pharmacological inhibition of specific signal transduction mediators on corneal wound healing using both monolayers of hCECs and the human tissue-engineered cornea (hTEC) as an in vitro 3D model. RNA and proteins were isolated from the wounded and unwounded hTECs to conduct gene profiling analyses and protein kinase arrays. The impact of WNK1 inhibition was evaluated on the wounded hTECs as well as on hCECs monolayers using a scratch wound assay. Gene profiling and protein kinase arrays revealed that expression and activity of several mediators from the integrin-dependent signaling pathways were altered in response to the ECM changes occurring during corneal wound healing. Phosphorylation of the WNK1 kinase turned out to be the most striking activation event going on during this process. The inhibition of WNK1 by WNK463 reduced the rate of corneal wound closure in both the hTEC and hCECs grown in monolayer compared with their respective negative controls. WNK463 also reduced phosphorylation of the WNK1 downstream targets SPAK/OSR1 in wounded hTECs. These in vitro results allowed for a better understanding of the cellular and molecular mechanisms involved in corneal wound healing and identified WNK1 as a kinase important to ensure proper wound healing of the cornea.

KEYWORDS

biomaterial, cornea, signal transduction pathway, tissue engineering, WNK1 kinase, wound healing

1 | INTRODUCTION

The eyes are one of the most precious sensory organs of the human body. They provide us vision, which is essential to perceive and interact with our surrounding environment. The functionality of the visual system relies on each structure composing the eye. One such particularly important structure is the cornea. The cornea forms the transparent anterior segment of the eye and accounts for three fourths of the refractive power of this organ (Eghrari, Riazuddin, & Gottsch, 2015). However, because of its superficial anatomical localization, it is particularly vulnerable to abrasive forces and various traumas. In the United States, the rate of eye injury exceeds 1 million each year, and it is estimated that approximately 20% of the population will experience eye injury at least once in their lifetime (Ljubimov & Saghizadeh, 2015). Approximately 75% of all eye injuries affect the cornea and are due to foreign bodies or abrasive damages. Other causes of corneal wounds include burns, punctures, and viral or bacterial infections (McGwin & Owsley, 2005). In general, successful wound healing

occurs through the renewal of corneal epithelial stem cells, but in some cases, particularly in the presence of a severe injury, or when the wound is not treated properly or rapidly, corneal injury may result in permanent visual impairment or lead to corneal blindness (Daniels, Dart, Tuft, & Khaw, 2001).

To investigate corneal wound healing, several models have been used over the years. Among others, the ex vivo culture organ model has been used to study healing of the cornea (Carrington & Boulton, 2005; Foreman, Pancholi, Jarvis-Evans, McLeod, & Boulton, 1996; Gipson & Anderson, 1980; Janin-Manificat et al., 2012; Zagon, Sassani, & McLaughlin, 2000). However, these studies are limited by the availability of healthy donors and the delay between donor's death and the arrival of the tissue in the laboratory. As for in vivo experiments, they are expensive and difficult to conduct because of the inherent variability among animals (Bentley et al., 2001; Buhren et al., 2009; Ferrington et al., 2013; Friedenwald & Buschke, 1944; Pallikaris, Papatzanaki, Stathi, Frenschock, & Georgiadis, 1990). Cell monolayers in vitro models are definitively less complex than the native cornea but can be of interest due to their ease and rapidity of use (Ker-Woon, Abd Ghafar, Hui, Mohd Yusof, & Wan Ngah, 2015; Lu, Wang, Dai, & Lu, 2010; Nelson, Silverman, Lima, & Beckman, 1990; Rieck, Cholidis, & Hartmann, 2001; Robciuc, Arvola, Jauhiainen, & Holopainen, 2018). Recent works in the field of tissue engineering have led to the emergence of new three-dimensional corneal equivalents. Some current approaches rely on the use of decellularized biological materials, as in the case of amniotic membrane and animal cornea (Ghezzi, Rnjak-Kovacina, & Kaplan, 2015). Others rely on the use of a variety of natural and synthetic polymers, such as collagen-chitosan, cross-linked recombinant collagen, and polyacrylamide or polyethylene glycol, in combination with primary derived corneal cells or immortalized corneal cell lines (Griffith et al., 1999; Reichl, Bednarz, & Muller-Goymann, 2004; Van Goethem et al., 2006). Using ascorbic acid to promote secretion of extracellular matrix (ECM) by fibroblasts in a procedure known as the self-assembly approach (Auger, Remy-Zolghadri, Grenier, & Germain, 2002; Germain, Carrier, Guérin, Salesse, & Auger, 2004), we succeeded in the reconstruction of a human, three-dimensional, tissue-engineered cornea (hTEC; Carrier et al., 2008; Germain et al., 1999; Proulx et al., 2010). When mechanically wounded, the hTEC triggers processes such as cell migration and proliferation, to allow re-epithelialization of the damaged tissue, which is found to be very similar to wound healing of a native cornea (Carrier et al., 2008; Couture et al., 2016; Zaniolo, Carrier, Guerin, Auger, & Germain, 2013).

During corneal wound healing, many processes such as cell migration, proliferation, differentiation, and adhesion will chronologically take part in the wound healing response. The first event is the migration of cells adjacent to the wounded area whose function is to seal the wound, followed by their proliferation and vertical differentiation in order to produce the stratified neoepithelium. The last event consists in the reassembly of adhesion structures (Agrawal & Tsai, 2003; Liu & Kao, 2015). Corneal wound healing relies on cell–cell and cell–matrix interactions, themselves mediated in great part by ECM components, but also by growth factors and cytokines (Nishida & Tanaka, 1996; Wilson et al., 2001; Zieske, 2001). Upon corneal epithelium injury, important changes occur in the composition of the ECM to which the basal corneal epithelial cells attach. These alterations are detected by integrins, a family of membrane-anchored receptors that trigger outside-in signaling between ECM and the cell (Hynes, 1987). Integrins act by recognizing ECM components as their ligand and subsequently activating different intracellular signaling pathways (Giancotti & Ruoslahti, 1999). Although it is now well established that intracellular signaling by integrins is a key step in the re-epithelialization process of wounded cornea, the specific mediators involved in that mechanism remain unknown.

In the present study, we used both monolayers of human corneal epithelial cells (hCECs) and three-dimensional human tissue-engineered corneas (hTECs) as in vitro models to investigate the impact of such ECM changes on the gene expression pattern and the activity of several intracellular signaling kinases during corneal wound closure. Phosphorylation of the serine/threonine protein kinase WNK1 (with no lysine (K) kinase 1) turned out to be the most striking activation event occurring during wound healing of damaged hTECs. Pharmacological inhibition of WNK1 by WNK463 prevents activation of its downstream targets Ste20/SPS1-related proline–alanine-rich protein kinase (SPAK) and oxidative stress responsive 1 (OSR1) kinases and considerably impedes the process of corneal wound healing in our biological models, suggesting that WNK1 plays a particularly important role in order to ensure proper healing of the cornea.

2 | MATERIALS AND METHODS

This study was conducted in accordance with our institution's guidelines and the Declaration of Helsinki. The protocols were approved by the CHU de Québec—Université Laval hospital and Université Laval Committees for the Protection of Human Subjects.

2.1 | Cell culture, production, and wounding of the tissue-engineered human corneas

hCECs were isolated from the limbal area of normal eyes of 44-year-old (hCEC-44), 52-year-old (hCEC-52), 70-year-old (hCEC-70X), and 71-year-old (hCEC-71) donors obtained from the Banque d'Yeux Nationale of the Centre Universitaire d'Ophtalmologie (CHU de Québec—Université Laval, Québec, QC, Canada). hCECs were cultured in the presence of a feeder layer of irradiated murine Swiss-3T3 fibroblasts, frozen and stored in liquid nitrogen until use for hTEC production, as previously reported (Carrier et al., 2008; Gaudreault et al., 2003; Germain et al., 1999; Germain et al., 2004). In the present study, new batches of hTECs were produced, wounded, and left to recover at the air–liquid interface using the same protocol as recently described (Couture et al., 2016). Briefly, hCECs were seeded on reconstructed stroma containing corneal fibroblasts, cultured 7 days in submerged conditions and 7 days at the air–liquid interface to induce epithelial cell differentiation and vertical stratification. Then hTECs were

wounded 14 days after the addition of epithelial cells. When indicated, the WNK1 inhibitor WNK463 (50 nM, 1 μM, or 10 μM; Novartis, Basel, Switzerland) was added to the culture medium of hTECs immediately after they were wounded with the biopsy punch. The specific concentrations selected for this pharmacological compound are based on MTS assays conducted on hCECs grown as a monolayer (see Supporting Information and Figure S1). Wound closure was then monitored macroscopically for 6 days and photographed at 24-hr intervals with a Zeiss Imager.Z2 microscope (Zeiss Canada Ltd., North York, ON, Canada). All experiments were conducted in quadruplicates except when indicated otherwise. Epithelial tissue from the central area and the external ring of both wounded and unwounded (used as negative controls) hTECs were harvested 4 days postwounding to collect both total RNA and proteins required for microarray and Western blot analyses, respectively, except for the SPAK/OSR1 Western that used total proteins from 6 days postwounding. hCEC-44, hCEC-52, and hCEC-71 were used for the preparation of hTECs used for gene profiling analyses, whereas hCEC-52 and hCEC-71 were used for the wound healing experiments conducted with the inhibitor WNK463.

2.2 | Cell cycle analysis

hCEC-52 and hCEC-70X (3×10^5 cells) were each plated with irradiated murine 3T3 fibroblasts (7.5×10^5 cells) in 9.6-cm^2 plates in DH medium (Dulbecco–Vogt modification of Eagle's medium with Ham's F12 in a 3:1 ratio) supplemented with 5% FetalClone II serum, 5 μg/ml of insulin, 0.4 μg/ml of hydrocortisone, 10 ng/ml of epidermal growth factor, 10^{-10} M of cholera toxin, 100 IU/ml of penicillin, and 25 μg/ml of gentamycin, and with (1 μM) or without (control [Ctrl] DMSO) WNK463. When cells reached confluence, hCECs were collected, fixed with 70% cold ethanol for 30 min, washed with phosphate-buffered saline (PBS), and stored at 4°C until use. Fixed cells were then treated with RNase A (100 μg/ml) and PI (50 μg/ml) at room temperature in the dark for 30 min. The DNA content of the cells was analysed using flow cytometry, and acquisition of data for 50,000 events was performed using a BD Accuri™ C6 Plus flow cytometer (BD Biosciences, San Jose, CA, USA). The distribution of hCECs within each phase of the cell cycle was analysed using the BD Accuri™ C6 Plus flow cytometry workstation software.

2.3 | Scratch wound assay

hCEC-52, hCEC-70X, and hCEC-71 (2.5×10^5 cells) were each plated in duplicates with irradiated murine 3T3 fibroblasts (7.5×10^5 cells) in 9.6-cm^2 plates in complete DH medium. When cells reached confluence, a 1-mm-large × 35-mm-long scratch was created in the centre of the plate using a P200 pipet tip (Sarstedt, Nümbrecht, Germany) prior to addition of either WNK463 (50 nM or 1 μM; Novartis) or DMSO (negative control) to fresh culture media. Wound closure was monitored, and photographs were collected at various time intervals (0, 5, 8, 9, 10, 11, 12, 13, 14, and 15 hr).

2.4 | Measurement of cells growth rate

hCEC-52 and hCEC-70X (2.5×10^5 cells) were plated with irradiated murine 3T3 fibroblasts (7.5×10^5 cells) in 9.6-cm^2 tissue-culture plates in complete DH medium. WNK463 (50 nM or 1 μM; Novartis) or DMSO (negative control) was added to fresh culture media every day. When cells reached confluence (or growth arrest), they were harvested and counted using a cell and particle counter (Z2; Beckman Coulter, Mississauga, ON, Canada). Cell counts were performed at passages (P) P3, P4, and P5. The growth rate per day was determined using the initial number of cells, final number of cells, and the duration of culture in days according the following formula:

$$\text{Growth rate} = \frac{\log((\text{final number of cells})/(\text{initial number of cells}))}{\log(2)} \Big/ \text{duration of culture in days}. \quad (1)$$

2.5 | Western blots

Western blots were conducted as described (Larouche, Leclerc, Salesse, & Guerin, 2000) using the following primary antibodies: rabbit polyclonal antibodies against phospho-WNK1 (1:250; Cell Signaling Technology Inc.; detects endogenous levels of WNK1 only when phosphorylated at Thr60), total WNK1 (1:250; Cell Signaling Technology Inc.), phospho-SPAK/OSR1 (1:250; Cell Signaling Technology Inc.; detects endogenous levels of SPAK and OSR1 when phosphorylated at Ser373 and Ser325, respectively), total SPAK (1:250; Cell Signaling Technology Inc.), total OSR1 (1:500; Cell Signaling Technology Inc.), actin (1:40,000; Santa Cruz Biotechnology, Dallas, TX, USA), and a peroxidase-conjugated AffiniPure Goat secondary antibody against mouse and rabbit IgG (1:2,500; Jackson ImmunoResearch Laboratories, Baltimore, PA, USA). The labelling was revealed using a Detection Kit (Amersham, Baie d'Urfé, Canada) as described (Gaudreault, Vigneault, Leclerc, & Guerin, 2007; Gingras et al., 2003).

2.6 | Immunofluorescence analysis

hCEC-52 cells were seeded in four-well culture inserts (Ibidi, Martinsried, Germany) set on coverslip glasses at 7×10^4 cells per well. Inserts were removed after 18 hr and cells maintained for 24 hr in complete DH medium supplemented with 1-μM WNK463 or DMSO (negative control). Cells were then fixed in 4% formaldehyde

for 20 min at room temperature and rinsed in PBS. Prior to immunodetection, cells were permeabilized with 0.2% Triton X-100 for 10 min. They were then incubated 30 min in the presence of phalloidin-Alexa 488 (1:200, Invitrogen, Carlsbad, CA, USA) and washed with PBS. Cell nuclei were counterstained with Hoechst reagent 33258 (1:100; Sigma). Fixed cells were observed with an epifluorescence microscope (Zeiss Imager.Z2; Zeiss Canada Ltd.) and photographed with a numeric CCD camera (AxioCam MRm; Zeiss Canada Ltd.).

2.7 | Gene expression profiling

Total RNA was isolated from epithelial cells isolated from the central area and the external ring of both wounded and unwounded (used as negative controls) hTECs using the RNeasy Mini Kit (QIAGEN, Toronto, ON, Canada) and its quality determined (2100 bioanalyzer, Agilent Technologies, Mississauga, ON, Canada) as recently described (Couture et al., 2016). Labelling of cyanine 3-CTP labelled targets, their hybridization on a G4851A SurePrint G3 Human GE 8 × 60K array slide (Agilent Technologies), and data acquisition and analyses were all done as previously reported (Couture et al., 2016; GSE # 113438). For statistical purpose, cell samples used for microarrays were obtained from both wounded and unwounded hTECs prepared using hCECs cultured from three different donors (hCEC-44, hCEC-52, and hCEC-71).

2.8 | Human phospho-kinase profiler array

The relative level of 43 different human phosphorylated protein kinases was determined using a membrane-based antibody array (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, equal amounts (300 μg) of cell lysates prepared from either the central or the external area of wounded hTECs were incubated overnight with the phosphokinase array membrane, washed to remove unbound proteins, and then incubated with a mixture of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were applied, and the signal produced at each captured spot was quantified using ImageJ (from Wayne Rasband, National Institute of Health, USA).

2.9 | Statistical analyses

Groups for both the scratch and hTECs wound healing analyses were compared using one-way analysis of variance with Tukey's multiple comparisons test, and $p < .05$ was considered significant. The sample size included data from three independent experiments performed in triplicate.

3 | RESULTS

3.1 | Impact of hTEC wound healing on the expression of mediators from the MAPK and PI3K/Akt pathways

We recently exploited gene profiling on microarray to examine the impact of wound healing on the expression of the major signal transduction mediators using, as a model, our hTECs. Alterations were observed in a number of mediators primarily from the MAPK and PI3K/Akt pathways (Couture, Desjardins, Zaniolo, Germain, & Guerin, 2018). However, our analysis only compared the pattern of genes expressed in the central re-epithelializing wound with the external area surrounding the initial wound in the hTECs. As important variations may occur in the expression of genes from the epithelial cells of the external ring due to a phenomenon described as the signal wave (Couture et al., 2016; Gao et al., 2013), we repeated these analyses by comparing the pattern of genes expressed in both the central and external areas of our wounded hTECs (Figure 1a) with that from unwounded hTECs. As shown on Figure 1b, an increase in the expression of MEK1, coordinated with a decrease in the expression of CREB and STAT5B, were the main changes observed in the mediators from the MAPK pathway. Interestingly, besides the decrease in CREB expression and the increased expression of the integrin adaptor kinase FAK, we also noted a significant increase in the expression of the WNK1 kinase, a mediator from the PI3K/Akt pathway (Figure 1b).

3.2 | hTEC wound healing alters the phosphorylation status of WNK1

As WNK1 is a downstream target along the PI3K/Akt signalization pathway, we next conducted phospho-kinase arrays in order to determine whether hTEC wound healing alters the phosphorylation level of WNK1 through the activation of this signalization route (Figure 2a). Interestingly, the most striking change in the phosphorylation status of the 43 kinases analysed by the array (Figure 2b) occurred for WNK1, whose phosphorylation level strongly increased in the central wound relative to its level in the epithelial cells from the external area (Figure 2c,d). Consistent with the increased WNK1 phosphorylation, we also observed a significant increase in the activation of Akt (a kinase that recognizes WNK1 as its target) in the central wound (Figure 2c,d). On the other hand, other protein targets along the PI3K/Akt pathway, such as CREB, HSP27, and Hck, became hypophosphorylated in the central wound (Figure 2c), suggesting that their activation may be detrimental to corneal wound healing.

To validate further the increased activation of WNK1 during wound healing, we conducted Western blot analyses in order to examine the level of total WNK1 (tWNK1) together with its phosphorylated counterpart (pWNK1) in the proteins extracted from both the central and external regions of our wounded

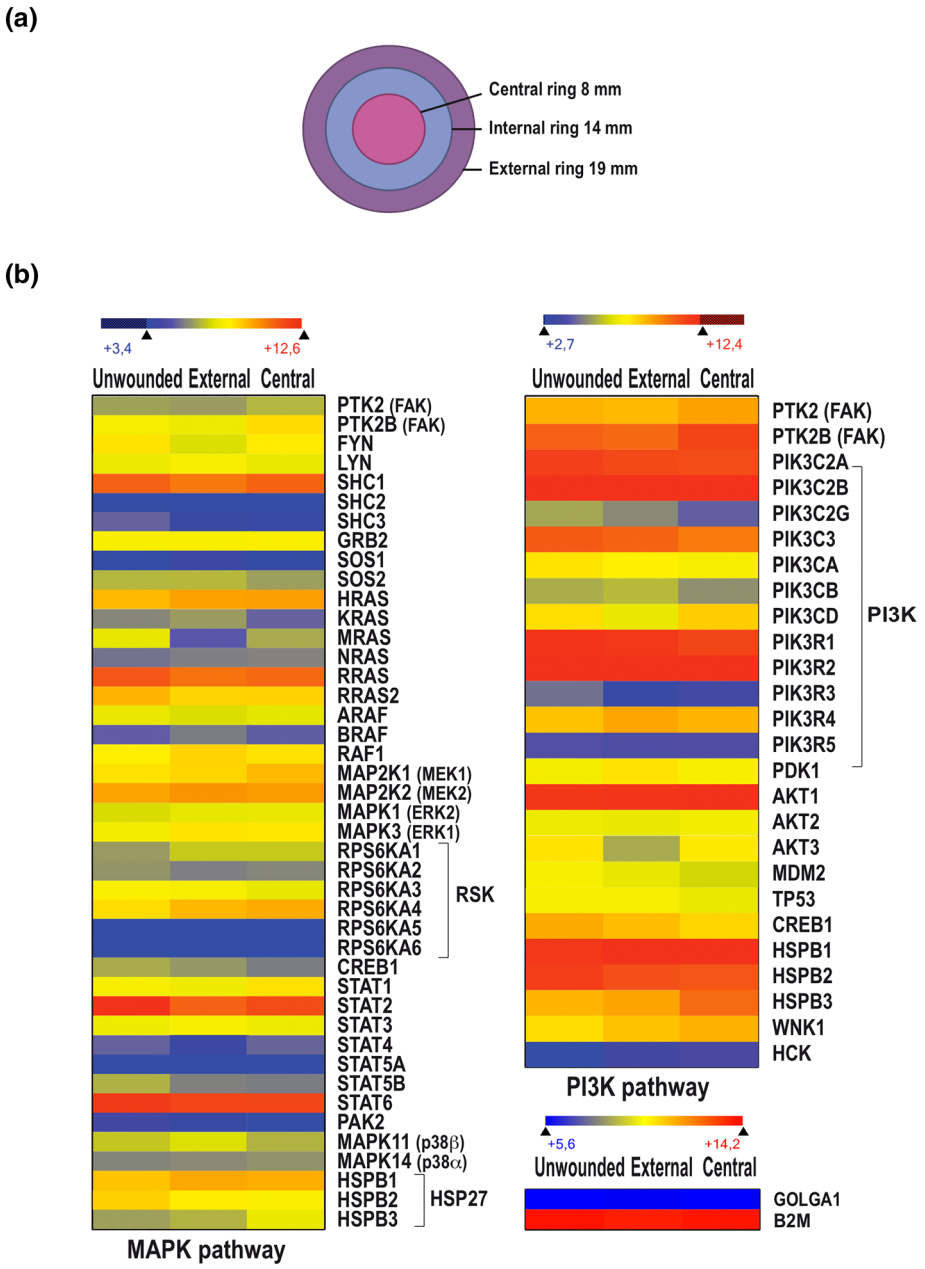

FIGURE 1 Gene profiling analysis of the mediators from the signal transduction pathways activated by wound healing in hTECs. (a) Schematic representation of the hTECs areas from which total RNA was extracted for microarray analyses. (b) Heatmap of the most important modulators from the MAPK and PI3K/Akt signal transduction pathways expressed by wounded (central, external) relative to their levels in unwounded hTECs (data shown correspond to the pooled microarray data obtained individually for hCEC-44, hCEC-52, and hCEC-71 cells). The colour scale used to display the log2 expression level values is determined by the hierarchical clustering algorithm of the Euclidian metric distance between genes. Genes indicated in orange/red correspond to those whose expression is high whereas very low expressed genes are shown in blue. Microarray data for the golgin subfamily A member 1 (GOLGA1) and β2-microglobulin (B2M) housekeeping genes that are expressed, respectively, to low and very high levels in all types of cells are also shown [Colour figure can be viewed at wileyonlinelibrary.com]

hTECs. A significant increase (2.5-fold) in the pWNK1/tWNK1 ratio was observed in the central wound compared with the external area of our wounded hTECs (1.16 vs. 0.47, respectively; Figure 2e). Therefore, activation of the PI3K/Akt pathway is coordinated with the phosphorylation of WNK1 during hTEC wound healing.

3.3 | Pharmacological inhibition of WNK1 delays hCECs and hTECs wound closure

As no study ever examined the impact of WNK1 phosphorylation on the dynamic of corneal wound healing, hCECs were therefore grown as monolayers and scratch wounded before they were added the

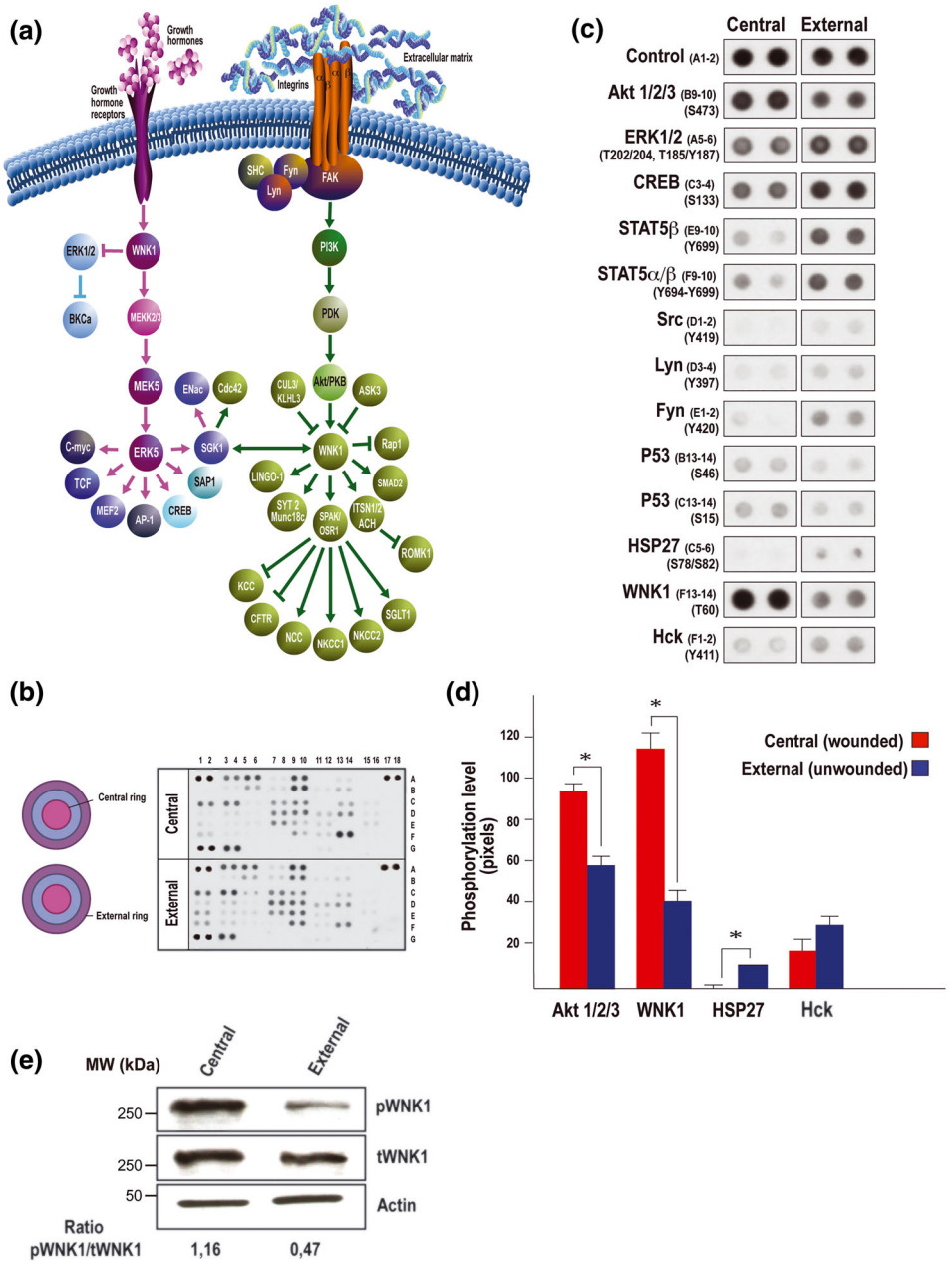

FIGURE 2 Kinases profiling analysis during corneal wound healing. (a) Major protein mediators from the WNK1 signal transduction pathways. (b) Cell lysates isolated from the central and external areas of hTECs assembled using the hCEC-44, hCEC-52, and hCEC-71 were pooled together and used for detection of activated kinases using the Human Phospho-Kinase Array from R&D Systems. (c) Kinases and mediators identified in B as being differentially phosphorylated between the central (wounded) and external (unwounded) areas of hTECs. (d) Densitometric analysis of the dot blot duplicates from panel (c) for the Akt, WNK1, HSP27, and Hck mediators between the central (red) and external (blue) areas of hTECs. (e) Cell lysates from the central and external areas of wounded hTECs were analysed by immunoblotting to confirm the phosphokinase array results for the mediator WNK1. Actin was used as the loading control [Colour figure can be viewed at wileyonlinelibrary.com]

WNK-specific inhibitor WNK463 (Yamada et al., 2016). As a control, scratch-wounded hCECs were also exposed solely to the vehicle (DMSO). Addition of 1-μM WNK463 to the culture medium of wounded monolayers of hCECs severely delayed closure of the wounds from 10 hr (in control DMSO and 50-nM WNK463) to 15 hr (1-μM WNK463; Figure 3a,b). We next stained the cells with phalloidin conjugated to Alexa Fluor 488 in order to selectively label F-actin in fixed, scratch-wounded hCECs. As shown on Figure 3c, at the edge of the wounded area, F-actin fibres are organized differently in control hCECs exposed only to DMSO than in cells grown in the presence of 1-μM WNK463 (cortical actin close to plasma membrane). In contrast, there are numerous lamellipodia (white arrows) in the control hCECs with actin present in these extensions, whereas nearly none can be observed in hCECs cultured in the presence of WNK463. This is consistent with the significant reduction in the migratory properties of hCECs in the presence of the WNK463 inhibitor.

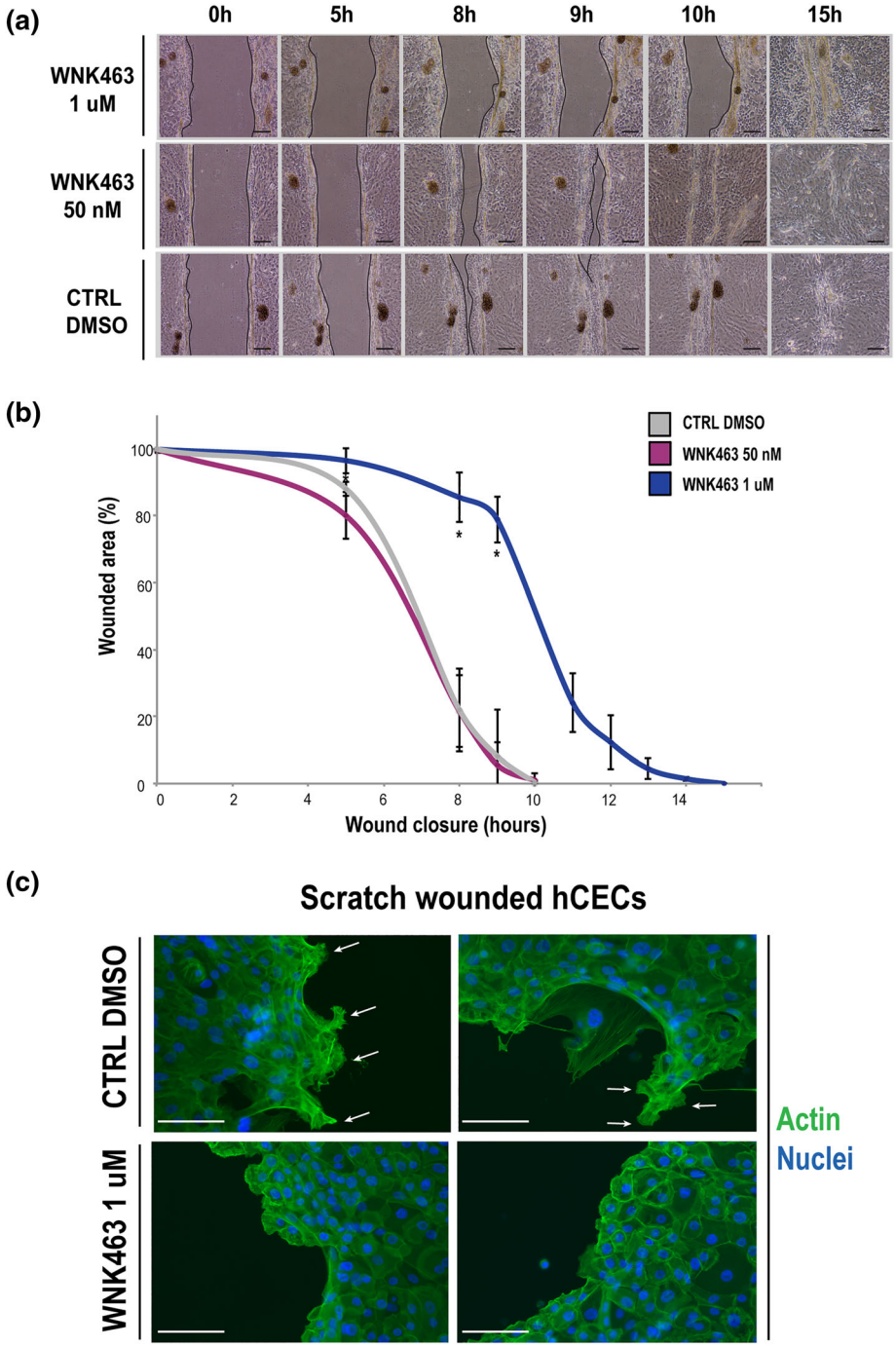

FIGURE 3 Impact of WNK1 inhibition by WNK463 on wound closure of hCECs. (a) Human corneal epithelial cells (hCEC-52, hCEC-70X, and hCEC-71) grown as a monolayer were wounded and allowed to recover in the presence of 50 nM or 1 μM of the WNK1 inhibitor WNK463. Scratches (4/condition) were photographed at various time intervals (0 to 15 hr) to monitor wound closure. As a negative control, hCECs were also incubated with the vehicle alone (DMSO). Scale bar: 200 μm. (b) Wound surfaces remaining for each condition were determined at each hour (time intervals from 0 to 15 hr) and plotted on graph. $^{*}p < .05$. (c) Phalloidin staining of actin filaments (green) in cells at the margin of the scratch wound made in hCEC monolayers grown in the presence of either 1-μM WNK463 or DMSO (left and right: two different fields of view). Arrows indicate the position of lamellipodia. Nuclei were counterstained with Hoechst 33258 reagent (blue). Scale bar: 100 μm [Colour figure can be viewed at wileyonlinelibrary.com]

As our hTECs are much closer from the native cornea than monolayers of hCECs can be, we therefore repeated this experiment on wounded hTECs as a model. Although 1-μM WNK463 almost completely suppressed wound healing when hCECs are grown as a monolayer, it proved insufficient at this concentration to alter wound closure in wounded hTECs, complete wound closure being achieved after 6 to 7 days in DMSO-treated hTECs and either 50-nM- or 1-μM-treated hTECs (Figure 4a,b). On the other hand, increasing

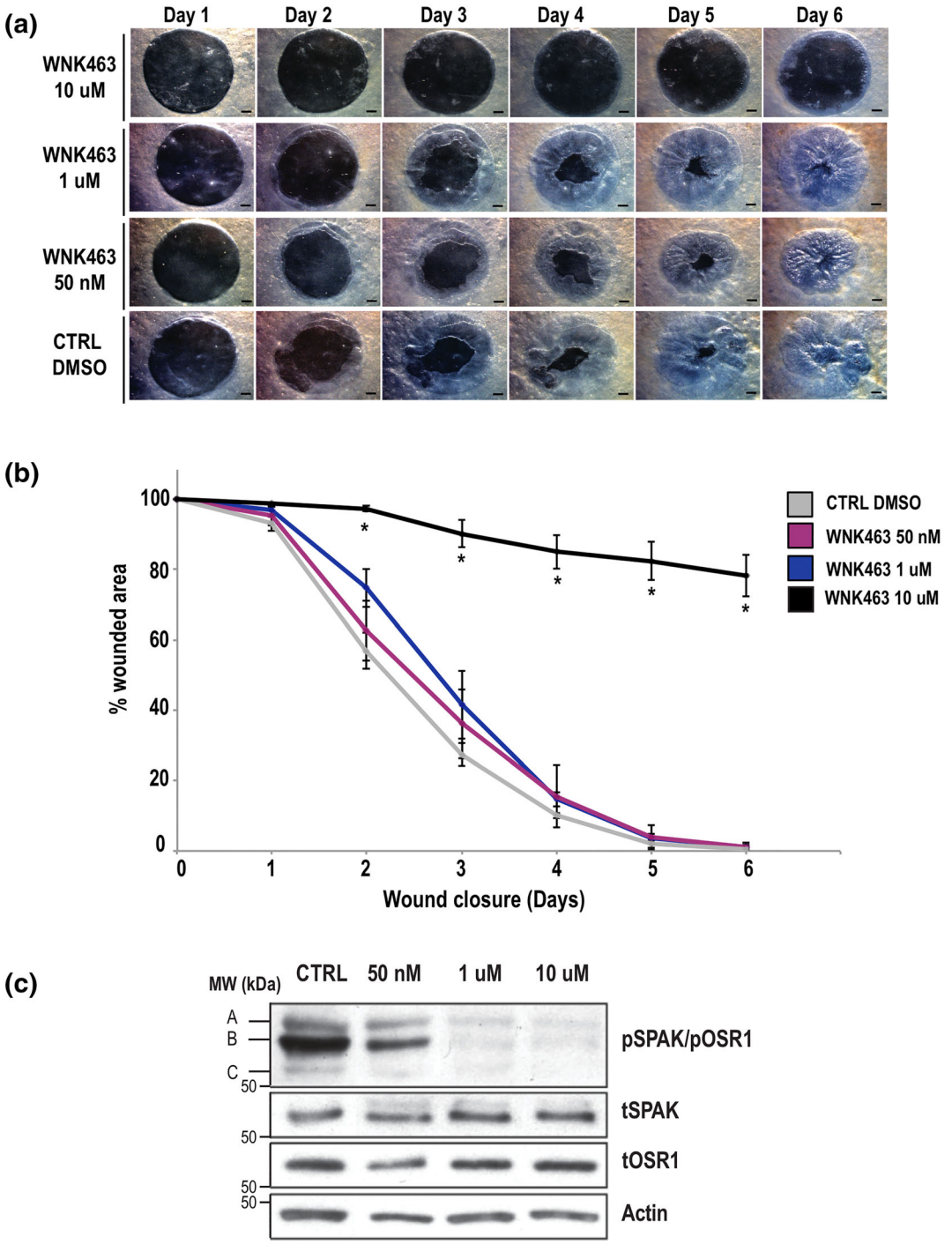

FIGURE 4 Impact of WNK1 inhibition by WNK463 on wound closure of human tissue-engineered cornea. (a) Human tissue-engineered corneas (hTECs) were wounded and allowed to recover in the presence of 50 nM, 1 μM, or 10 μM of the WNK1 inhibitor WNK463. Corneas (4/condition) were photographed at each day (Days 1 to 6) to monitor wound closure. As a negative control, hTECs were also incubated with the vehicle alone (DMSO). Scale bar: 1 mm. (b) Wound surfaces remaining for each condition were determined at each day and plotted on graph. $^{*}p < .05$. (c) Cell lysates from the external areas of wounded hTECs exposed (50 nM, 1 μM, or 10 μM) or not (Ctrl) to WNK463 were used as source of biological materials in order to monitor the expression of the total (tSPAK and tOSR1) and phosphorylated (pSPAK and pOSR1; Bands A to C) WNK1 downstream target mediators SPAK and OSR1 by Western blot. Actin was used as the loading control [Colour figure can be viewed at wileyonlinelibrary.com]

further the concentration of WNK463 to 10 μM almost entirely abolished wound closure as more than 80% of the wounded area still remains after 6 days in culture.

To further validate the inhibition of WNK1 when wounded hTECs are exposed to the WNK463 inhibitor, we conducted Western blot analyses in order to examine both the WNK1 downstream target

proteins SPAK (tSPAK) and OSR1 (tOSR1; Moriguchi et al., 2005; Vitari, Deak, Morrice, & Alessi, 2005), together with their phosphorylated counterpart (pSPAK/pOSR1) in the proteins extracted from the external region of our wounded hTECs (no sample from the central, wounded area was used as 10-μM WNK463 prevented wound closure). As shown on Figure 4c, both total (tSPAK and tOSR1) and phosphorylated SPAK/OSR1 (pSPAK/pOSR1; they appear as three distinct protein bands: A, B, and C) could easily be detected in wounded hTECs that have not been exposed to the WNK463 inhibitor (Ctrl). On the other hand, exposure of hTECs to 50-nM WNK463 resulted in a significant reduction in the phosphorylation of both SPAK and OSR1, whereas nearly no signal is observed when 1- or 10-μM WNK463 is used. The presence of WNK463 had no significant impact on either total SPAK (tSPAK) or OSR1 (tOSR1). We therefore conclude from these results that inhibition of WNK1 by WNK463 dramatically reduces the efficiency of wound closure in our hTECs.

3.4 | Inhibition of WNK1 alters the proliferative properties of hCECs

We next evaluated the impact of the WNK1 inhibitor on the proliferative properties of two different populations of hCECs by measuring their growth rate upon cell passages when cultured as monolayers. As shown on Figure 5a, cell's growth rate progressively diminished (from 0.52 at P3 to 0.35 at P5) as hCECs are passaged in culture in the absence of WNK463 (Ctrl DMSO). Yet they could still form colonies of small epithelial cells after 18 days at P5 (Ctrl; Figure 5b). Identical results were also obtained when hCECs are grown in the presence of 50-nM WNK463 (Figure 5a,b). On the other hand, incubation of hCECs in the presence of 1-μM WNK463 resulted in a significant reduction of the hCEC's growth rate from 0.52 (control) to 0.25 (1-μM WNK463) at P3, whereas this value dropped dramatically at P4, from 0.42 (control) to only 0.03 (1-μM WNK463; Figure 5a). After only 5 days in culture at P3, hCECs exposed to 1-μM WNK463 grew in very small colonies surrounded by i3T3 and preserved that morphological characteristic at P4 even when maintained in culture for 18 days, unlike control samples whose much larger colonies easily displaced the i3T3 feeder cells under phase contrast microscopy (Figure 5b). hCECs grown at P4 in the presence of 1-μM WNK463 could not be cultured further at P5. To determine whether the drastic change in hCEC's growth rate when these cells are cultured in the presence of WNK463 might have resulted from alterations in the cell cycle progression that also led these cells into terminal differentiation, hCECs grown with 1-μM WNK463 for 10 days at P3 were harvested and fed fresh culture medium without inhibitor and allowed to grow further at P4 for five and eight more days in culture. As Figure 5a indicates, WNK463-arrested hCECs (1-μM WNK463) could easily be rescued by removing the inhibitor from the culture medium (1μM - ø of WNK463), the cell's growth rate increasing from 0.03 to 0.37, a value very similar to that measured for control hCECs (0.42). In addition, replacing the WNK463-containing medium with fresh DH medium also restored the ability of the colonies of hCECs to expand again normally after 5 (total of 15 culture days) or 8 days (total of 18 culture days) under inhibitor-free condition (Figure 5b). Cell cycle analysis of hCECs grown at P4 in the presence of WNK463 indicates that addition of the WNK1 inhibitor caused more cells to be arrested in both the G0/G1 (61% of cells arrested compared with 54% in DMSO [control]) and S phases (18% of cells arrested compared with 14% in DMSO [control]) of the cell cycle, as a result of a decreased number of cells in the G2/M phase (21% vs. 32% for WNK463- and DMSO-treated cells, respectively; Figure 5c).

4 | DISCUSSION

Injury to the corneal epithelium alters the composition of the ECM to which corneal epithelial cells are bound and thereby triggers integrin-mediated activation of different intracellular signaling pathways that each participate to the wound healing process. Although the overall aspects of the signalization cascades involved in that process have been intensively investigated, the precise mechanistic details by which they contribute to corneal wound healing have yet to be determined. By exploiting both monolayers of hCECs and hTECs as in vitro models, we have shown that phosphorylation-mediated activation of the WNK1 kinase was one particularly important event occurring during hTEC wound healing. The especially important contribution of this kinase to the wound healing process was demonstrated by the inability of wounded hTECs to properly heal upon pharmacological inhibition of WNK1 by WNK463, a process that is likely mediated by the WNK1 downstream target mediators SPAK/OSR1.

We recently demonstrated that hypophosphorylation of CREB and activation of Akt was particularly important to ensure proper closure of wounded hTECs in vitro (Couture et al., 2018). However, in the present study, we showed that besides these two signal transduction mediators, corneal wound healing also induces a strong phosphorylation of the WNK1 kinase, a mediator that can be activated via multiple routes such as the JAK/STAT, MAPK, or the PI3K/Akt pathways (Figure 2a). The change observed in the expression/activation status of WNK1 was confirmed by Western blot analysis of its phosphorylated-over-total protein ratio. The contribution of WNK1 to corneal wound healing must be particularly important as addition of 10-μM WNK463 to punch-wounded hTECs dramatically inhibited wound closure in a reversible manner. WNK1 is the founding member of a family that comprises four evolutionarily conserved serine–threonine kinases (WNK1, WNK2, WNK3, and WNK4) that share 85% homology over their kinase domains (Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002). Activation by phosphorylation of WNK kinases allows them to respond to changes in intracellular chloride concentration (Cl^-) and tonicity (Richardson & Alessi, 2008; Zagorska et al., 2007). Whereas expression of WNK2 and WNK3 is usually restricted to the kidney, that of WNK1 is ubiquitous (reviewed in Rodan & Jenny, 2017). In agreement with these results, unwounded hTECs were found to express intermediate levels of the WNK1 mRNA transcript and only low levels of WNK2 and WNK4 whereas no

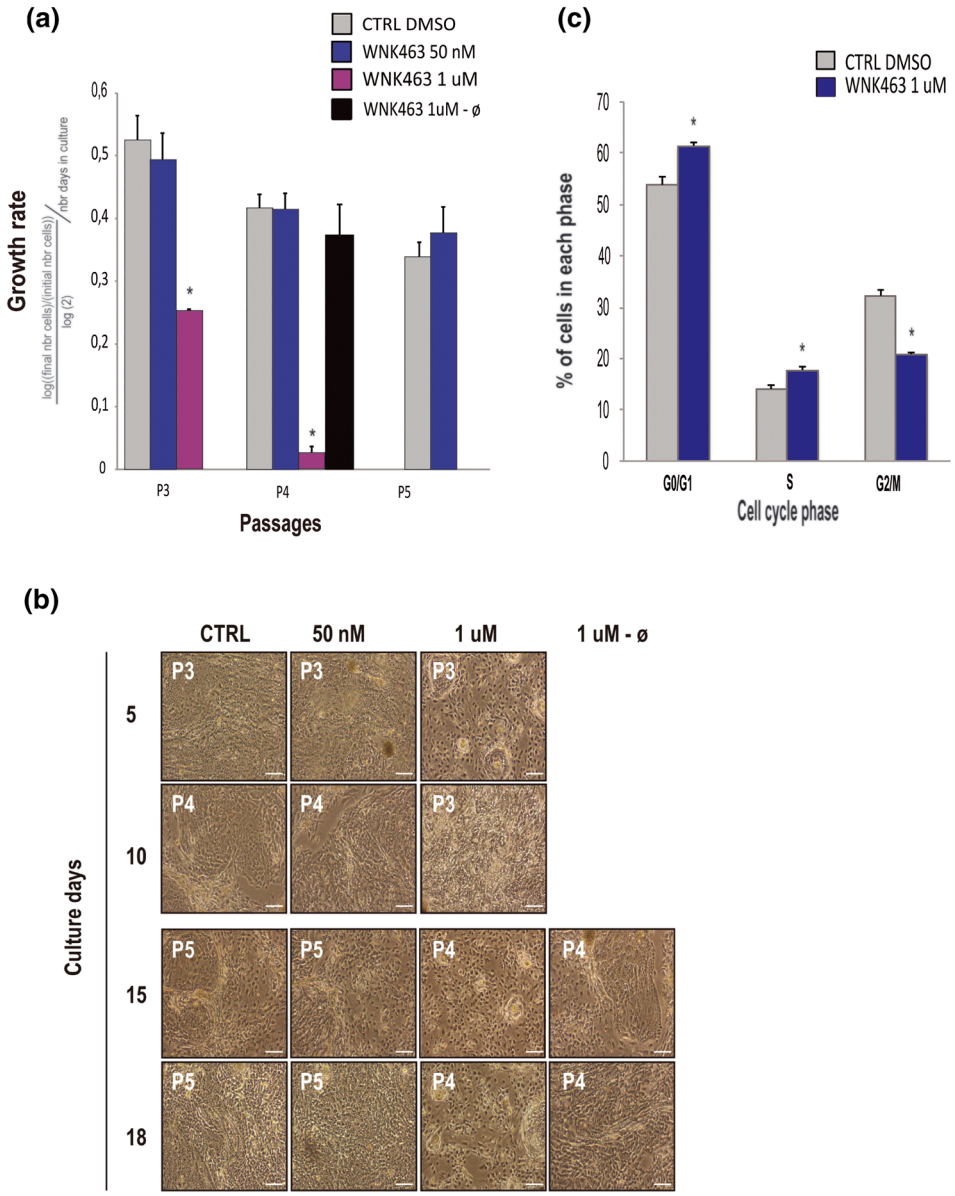

FIGURE 5 Impact of WNK463 on the proliferative properties of hCECs in monolayer culture. (a) hCECs at P3 were grown as monolayers in either the absence (Ctrl DMSO) or the presence of the WNK1 inhibitor WNK463 (50 nM or 1 μM). When they reached complete confluence (or growth arrest), cells were harvested, counted to determine their growth rate, and grown further at P4 and P5. hCECs at P3 were also grown until they reached P4 in the presence of WNK463 (1 μM) and were maintained at P4 for 8 days. The culture medium was then changed for normal DH medium without any WNK1 inhibitor, and cells were allowed to grow for eight additional days (WNK463 1μM - ø) before they were harvested for determination of the cell's growth rate. hCECs growth rate was expressed as doubling time per day for each successive passage. $^{*}p <$ .05. (b) Phase contrast photographs of hCECs grown either without (Ctrl) or in the presence (50 nM, 1 μM) of WNK463 for 5, 10, 15, or 18 days in culture at P3 to P5; 1 μM − ϕ: photographs of hCECs grown with 1-μM WNK463 and rechallenged with complete DH medium in the absence of inhibitor. Scale bar: 200 μm. (c) Graphs showing the proportion of hCECs (expressed as %) grown with (1 μM) or without (Ctrl DMSO) WNK463 in each phase of the cell cycle (G0/G1, S, and G2/M). $^{*}p < .05$ [Colour figure can be viewed at wileyonlinelibrary.com]

expression of WNK3 was observed (Figure S2). Interestingly, whereas transcription of WNK1 increased in the central wound relative to unwounded hTECs, that of both WNK2 and WNK4 gradually disappeared (Figure S2). The reduced expression of WNK2 as wound healing is proceeding is somehow consistent with the observation that SW1088 glioblastoma cell line in which expression of this kinase was suppressed could form colony on soft agar and also had increased migratory properties in scratch wound healing assays (Moniz et al., 2013).

Our results are particularly interesting in that they suggest a novel function for WNK1 during corneal wound healing. WNK1 was recently reported as a negative regulator of integrin-mediated adhesion, whereas it acts as a positive regulator of migration via the kinases OSR1 (OXSR1) and SPAK (STK39) and the ion cotransporter NKCC1

(SLC12A2) in T cells (Kochl et al., 2016). To our knowledge, no study ever reported the expression of WNK1 in the cornea. However, its expression has been reported in other eye structures. Indeed, studies that described a role for the WNK1-SPAK/OSR1 pathway in the maintenance of the rodent, bovine, and human eye lens transparency have been recently published (Vorontsova et al., 2017; Vorontsova, Lam, Delpire, Lim, & Donaldson, 2015). Furthermore, participation of WNK1 to chloride accumulation and for the generation of calcium-dependent chloride current in rod photoreceptors has also been demonstrated (Dauner, Mobus, Frings, & Mohrlen, 2013). Besides the eye, WNK1 expression has been observed in a variety of epithelia, including the skin (Choate, Kahle, Wilson, Nelson-Williams, & Lifton, 2003). Our results raise the interesting hypothesis that as with the corneal endothelial cells, signalization through the WNK1-SPAK/OSR1 pathway may contribute at preventing swelling and loss of transparency of the corneal stroma during wound healing, which would be a remarkably novel finding as it would demonstrate a relationship between WNK1 signalization and wound healing in the cornea.

Inhibition of WNK1 by WNK463 also strongly reduced the phosphorylation level of both SPAK1 and OSR1 (phosphorylated SPAK and OSR1 could be simultaneously detected with an antibody that recognizes phosphorylation of the C-terminal regulatory domain common to both kinases; Saritas et al., 2013), two immediate downstream targets of WNK1. As for WNK1, this is the first demonstration that both SPAK and OSR1 are expressed in hCECs. A participation of SPAK to wound healing is well documented and not surprising considering the important role it has been shown to play in cell migration and adhesion. Indeed, mutation of SPAK has been shown to reduce intestinal epithelial cell/cell adhesion (Yan, Nguyen, Dalmasso, Sitaraman, & Merlin, 2007). In addition, a direct interaction of the complement modulator CD46 with SPAK was shown to induce cell proliferation and to accelerate wound healing of Caco-2 intestinal epithelial cells thereby suggesting a role for SPAK in the regulation of the epithelial cell barrier integrity and repair (Cardone, Al-Shouli, & Kemper, 2011). More recently, results by Y. Liu et al. (2018) suggested that SPAK activation, through insulin-induced chemotaxis of THP-1 cells, may also contribute to insulin-driven wound healing.

Our experiments demonstrated that a concentration of WNK463 higher (10 μM) than that used on the monolayer model (1 μM) was required to inhibit wound closure of punch-wounded hTECs. We believe there might be two explanations for this result: (a) Unlike for hCECs grown as a monolayer, which are directly exposed to WNK463, this pharmacological inhibitor, on the other hand, must diffuse through the reconstructed stroma of our hTECs before it reaches the layer of epithelial cells as this reconstructed tissue is fed with culture medium from the underneath. (b) Unlike when grown as a monolayer, hCECs that are grown on the hTECs are lying on a complex ECM whose many constituting components likely activate signal transduction pathways (such as the MAPK pathway) that share mediators also used by the WNK1 pathway as well. We recently demonstrated that addition of both the CREB inhibitor C646 and the Akt agonist SC79 to wounded hTECs considerably increased wound closure irrespective of whether it is deposited beneath (Couture et al., 2018) or over (unpublished results) the reconstructed tissue. It is therefore unlikely that addition of WNK463 underneath the hTEC accounts for the difference observed, which therefore rather favour an impact from the ECM. However, additional experiments will need to be conducted to determine whether this mechanism indeed accounts for the WNK463-dependent variations observed between the monolayer and hTEC models. Moreover, these results show the impact of WNK1 inhibition on corneal wound healing in a human context. The hTEC, however, bears the limitations of in vitro models. Because in vivo animal models may have some compensation mechanisms against pharmacological inhibition that are absent in vitro, further studies should be done in the future to evaluate whether these findings can be confirmed also in vivo.

Along with WNK1, we also noted an increase in the expression of SPAK at the transcriptional level in wounded hTECs, but not OSR1 (Figure S2). Similarly, transcription of the gene encoding the β subunit of the epithelial sodium channel ENaC is also increased in our wounding hTECs. However, of the many mediators from the WNK1/SPAK/OSR1 signalization axis, it is the transcript encoding cystic fibrosis transmembrane conductance regulator (CFTR) that is subjected to the most important alteration during hTEC wound healing. Indeed, and unlike for WNK1 and SPAK whose expressions increase in the central wound, that of CFTR is repressed by a factor of five (Figure S2). Although this might sound surprising at first, it is however consistent with the observation that both WNK1 and WNK4 are negative regulators that function in an additive manner to reduce CFTR abundance at the cell membrane (Yang et al., 2007).

Our results clearly suggest that activation of WNK1 is a feature of the hTEC wound healing process. How, then, might the activation of the WNK1/SPAK/OSR1 pathway contribute to wound healing? The answer to this puzzling question might come from the recent demonstration that activated WNK1 is a key participant in order to ensure proper balance between adhesion and migration in T cells. Indeed, activation of WNK1 through the PI3K/Akt pathway was found to negatively regulate integrin-dependent T-cell adhesion while improving their migratory properties (Kochl et al., 2016). It is the activation of the WNK1 downstream pathway OSR1–SPAK–NKCC1 that has been demonstrated to regulate cell migration likely through a mechanism involving ions transport across the cell membrane. This interesting hypothesis suggests that polarization occurs at the leading edge through an *Slc12a* Na–K–Cl cotransporter-dependent uptake of ions and water, whereas these are released at the trailing edge of the migrating cells, a process that would cause cell movement and described as the "osmotic engine model" (Cuddapah & Sontheimer, 2011; Stroka et al., 2014). The decrease in cell migration is also likely to be linked to the differences visualized in the organization of the actin network.

In summary, we demonstrated that activation of the WNK1-SPAK/OSR1 pathway is required to ensure proper wound closure of wounded tissue-engineered corneas in vitro. Activation of this signal transduction pathway during corneal wound healing raises the interesting possibility that ions and water transport in addition to actin reorganization might contribute to the proliferative and

adhesive/migratory properties of the corneal epithelial cells located nearby the wounded area. Animal studies will be performed in the future to validate our in vitro results and to evaluate whether compensation mechanisms against the pharmacological inhibition are present in the in vivo model.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. Gaëtan Le-Bel for his technical assistance. This work was supported by the Canadian Institutes for Health Research (CIHR) Grant MOP-53170 and FDN-143213 (L. G.), the Fondation des Pompiers du Québec pour les Grands Brûlés (FPQGB), the Fonds de Recherche du Québec-Santé (FRQS), and the Réseau de thérapie cellulaire, tissulaire et génique (ThéCell), a thematic network supported by the FRQS. The Banque d'yeux Nationale is partly supported by the Réseau de Recherche en Santé de la Vision, a thematic network supported by the FRQS. P. D. and C. C. were supported by studentships from the FRQS. L. G. is the recipient of a Tier 1 Canada Research Chair on Stem Cells and Tissue Engineering and a Research Chair on Tissue-Engineered Organs and Translational Medicine of the Fondation de l'Université Laval.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. MTS assays. Corneal epithelial cells grown as monolayers were exposed to increasing doses of WNK463 (in the 'x' axis; 1 nM to 50 μM). Cell viability (in the 'y' axis) was obtained by measuring the absorbance of formazan at 490 nm. The IC50 reported by Yamada et al. to inhibit phosphorylation of MBP (5 nM) and OSR1 (106 nM) by WNK1 in HEK293 cells are indicated in white and grey, respectively, on the graph (Yamada et al., 2016).

Figure S2. Microarray analysis of WNK1-activated mediators gene expression patterns in response to corneal damage. Heatmap representation of the expression profiles of the genes coding for known WNK1 target mediators between unwounded (control) and both the central and external area of wounded hTECs. The color scale used to display the log2 expression level values is determined by the Hierarchical clustering algorithm of the Euclidian metric distance between genes. Genes indicated in dark blue correspond to those whose expression is very low whereas highly expressed genes are shown in orange/red.

Table S1. Biological functions of the proteins encoded by the differentially expressed genes

Data S1. MTS Assays

How to cite this article: Desjardins P, Couture C, Germain L, Guérin SL. Contribution of the WNK1 kinase to corneal wound healing using the tissue-engineered human cornea as an in vitro model. *J Tissue Eng Regen Med*. 2019;1–14. https://doi.org/10.1002/term.2912